

AD\_\_\_\_\_

Award Number: DAMD17-99-1-9228

TITLE: Characterization of the Contribution of Ceramide to  
Chemotherapy Sensitization in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Hongtao Wang, M.D., Ph.D.  
Myles C. Cabot, Ph.D.

CONTRACTING ORGANIZATION: John Wayne Cancer Institute  
Santa Monica, California 90404

REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are  
those of the author(s) and should not be construed as an official  
Department of the Army position, policy or decision unless so  
designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	September 2000	Annual Summary (1 Sep 99 - 31 Aug 00)	
4. TITLE AND SUBTITLE		5. FUNDING NUMBERS	
Characterization of the Contribution of Ceramide to Chemotherapy Sensitization in Breast Cancer Cells		DAMD17-99-1-9228	
6. AUTHOR(S)		8. PERFORMING ORGANIZATION REPORT NUMBER	
Hongtao Wang, M.D., Ph.D. Myles C. Cabot, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
John Wayne Cancer Institute Santa Monica, California 90404			
E-MAIL: CabotM@jwci.org		12a. DISTRIBUTION / AVAILABILITY STATEMENT	
		Approved for public release; distribution unlimited	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		12b. DISTRIBUTION CODE	
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			
11. SUPPLEMENTARY NOTES			
13. ABSTRACT (Maximum 200 Words)			
<p>Our previous studies show that the drug resistance modulator, PSC 833, an analog of cyclosporin A, increases cellular ceramide levels, thus initiating caspase-apoptotic signaling. The mechanism of PSC 833 induced ceramide generation is, however, unknown. In order to use ceramide targeting as a therapeutic approach to chemotherapy sensitization in breast cancer, mechanism information is essential. To this end, during the past year, I have mastered techniques of lipid analysis, developed and standardized enzyme assays for ceramide synthase, serine palmitoyltransferase, and palmitoyl coenzyme A synthetase, and utilized human breast cancer cell lines in model systems. Our studies demonstrate that PSC 833 induces ceramide generation via the <i>de novo</i> biochemical pathway, as opposed to degradation of sphingomyelin. We have shown, for the first time, that serine palmitoyltransferase, and not ceramide synthase or palmitoyl coenzyme A synthetase is activated by PSC 833 in breast cancer cells. Furthermore, we also demonstrate a close structure-activity relationship for activation of serine palmitoyltransferase, based on studies with analogs of cyclosporin. This is a significant finding which sets the stage for continued drug studies aimed at targeting ceramide metabolism to enhance chemotherapy response in the treatment of breast cancer.</p>			
14. SUBJECT TERMS		15. NUMBER OF PAGES	
Breast Cancer		10	
20010302 059		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

## Table of Contents

1.	<b>Cover</b> .....	1
2.	<b>Form SF 298</b> .....	2
3.	<b>Table of Contents</b> .....	3
4.	<b>Introduction</b> .....	4
5.	<b>Body</b> .....	5-9
	<b>Training</b> .....	5
	<b>Research Accomplishment</b> .....	5-7
	<b>Conclusion</b> .....	7
	<b>References</b> .....	8-9
7.	<b>Appendix</b> .....	10
	<b>Key Research Accomplishments</b> .....	10
	<b>Reportable Outcome</b> .....	10

#### 4. INTRODUCTION

Multidrug resistance (MDR) is a major cause of treatment failure in breast cancer. The purpose of this research is to improve the efficacy of treatment. While P-glycoprotein (P-gp) may be the most understood mechanism of MDR (1-4), other important mechanisms have been identified in recent years, such as cellular increases in glucosylceramide, and changes in the activity of glutathione S-transferase, and topoisomerase (5-10). Numerous agents have been studied in an effort to overcome MDR (11-14). A major challenge in breast cancer chemotherapy today is to understand the molecular mechanisms by which MDR modulators, e.g. tamoxifen, PSC 833, reverse drug resistance (13,14). While studies have shown MDR modulators bind directly to P-gp and thus interfere with binding and export of anticancer agents, it is increasingly apparent that some chemotherapeutic agents stimulate ceramide generation in cancer cells, and this leads to apoptosis (15,16). Adriamycin, the most widely used single agent for treatment of breast cancer, also activates ceramide formation (17). Our recent studies show that PSC 833 markedly enhances cellular ceramide formation and is synergistic with adriamycin (14,17,18). Because the effect of PSC 833 on ceramide formation is correlated with an increase in cell death and reversal of multidrug resistance in breast cancer cells (14,18), knowledge of the biochemical pathways involved is essential. Ceramide generation may be increased by either hydrolysis of membrane-resident sphingomyelin or *de novo* synthesis at the endoplasmic reticulum (19-22). We will characterize the biochemical mechanism of action of PSC 833 on cellular ceramide metabolism using PSC 833 as a tool, together with conventional drugs like adriamycin and taxol. This will establish the contribution of ceramide to chemotherapy sensitization in breast cancer, and set the stage for alternative modes to treat resistant disease.

## 5. BODY

### Training-

- 1. Metabolic labeling of cellular lipids and thin-layer chromatographic analysis.** During the past year, I have mastered various techniques of lipid biochemistry, including radiolabeling of cellular lipids and thin-layer chromatography (TLC) for analysis of ceramide, glucosylceramide, and sphingomyelin. I have worked out TLC solvent systems for resolution of 3-ketoshinganine, sphinganine, and sphingosine. Using these techniques, I have shown that PSC 833 induces ceramide generation via *de novo* synthesis, and that an enzyme upstream of ceramide synthase is activated, namely serine palmitoyltransferase (SPT). Furthermore, I also have learned techniques useful to distinguish between ceramide generated *de novo* versus by sphingomyelin hydrolysis.
- 2. Enzyme Assays.** Several enzymes, such as palmitoyl coenzyme A synthetase, serine palmitoyltransferase, and ceramide synthase, are involved in *de novo* synthesis of ceramide. Test tube assays are useful to clarify the enzymes responsible for PSC 833 induced ceramide generation. However, *in vitro* assays for palmitoyl coenzyme A synthetase, serine palmitoyltransferase, and ceramide synthase have not developed for any human breast cancer cell lines. During the past year, I have successfully developed and standardized assays for these enzymes. The kinetics have been established to be linear with regard to protein concentration (enzyme) and time, and experiments are conducted under saturating substrate conditions. Establishing these enzyme assays in breast cancer cells was crucial to the project goals, and the progress was key to our accomplishments.

### Research Accomplishments-

- 1. PSC 833 induces apoptosis and reverses multidrug resistance in breast cancer cells.** PSC 833 induces apoptosis in both MDA-MB 468 and in MCF-7 breast cancer cell lines in a dose-dependent manner. PSC 833 also reverses resistance to doxorubicin in MCF-7/AdrR cells, a breast cancer cell line resistant to adriamycin.
- 2. PSC 833 increases ceramide generation in a time- and dose-dependent manner.** PSC 833 increased ceramide levels in MDA-MB 468 cells as early as 15 min after addition, and at a concentration as low as 0.1 $\mu$ M. Similarly, PSC 833 treatment elicited ceramide formation in MCF-7 cells. Use of either L-cycloserine, a potent inhibitor of SPT, or FB<sub>1</sub>, a specific inhibitor of ceramide synthase, retarded the generation of ceramide and reversed the effects of PSC 833 on modulation of drug resistance. These results indicate that ceramide is linked to the cytotoxic response elicited by PSC 833.
- 3. PSC 833 increases ceramide generation via the *de novo* pathway and not through sphingomyelin hydrolysis.** To determine if PSC 833 is effecting ceramide metabolism via *de novo* synthesis, the influence of FB<sub>1</sub> and L-cycloserine were evaluated. Pretreating MDA-MB 468 cells with FB<sub>1</sub> severely inhibited PSC 833-induced

ceramide formation, and pretreatment with L-cycloserine completely blocked the effects of PSC 833 on ceramide generation. These results show that PSC 833 induces ceramide generation via *de novo* synthesis.

To determine whether PSC 833 also influences sphingomyelin hydrolysis, and thus contributes to the ceramide pool, we prelabeled cells with [<sup>3</sup>H]palmitic acid for 24 hr. Treatment of prelabeled cells with PSC 833 revealed that the amount of [<sup>3</sup>H]ceramide generated from *de novo* pathway was 98.3% of the total amount of [<sup>3</sup>H]ceramide generated by both *de novo* synthesis and by hydrolysis of sphingomyelin. Therefore, sphingomyelin is not a source of PSC 833-induced ceramide formation.

**4. PSC 833 activates cellular serine palmitoyltransferase.** Pretreatment of MDA-MB 468 cells with PSC 833 followed by subcellular fractionation and isolation of microsomes, increased the activity of serine palmitoyltransferase by 180% when evaluated in cell-free assays. The effects of PSC 833 pretreatment on serine palmitoyltransferase activity were time- and dose-dependent. Pretreatment of MCF-7 and MCF-7/ArdR cells with PSC 833 also increased serine palmitoyltransferase activity. The increase in enzyme activity was detected as early as 30 min after PSC 833 exposure, and the activity increased linearly through 6 hr of pretreatment.

We also designed experiments to measure the activity of serine palmitoyltransferase in intact cells. MDA-MB 468 cells were radiolabeled with [<sup>3</sup>H]palmitic acid, a precursor to the serine palmitoyltransferase substrate palmitoyl CoA, and simultaneously treated with PSC 833. Sphinganine, the product of serine palmitoyltransferase, was measured by TLC. The results showed that sphinganine increased by 146 % at 6 hr after the addition of PSC 833 to the culture medium. Addition of FB<sub>1</sub> to block conversion of sphinganine to ceramide, increased PSC 833-induced generation of sphinganine by 186 %. These observations further support the finding that serine palmitoyltransferase is activated by the MDR modulator, PSC 833.

**5. PSC 833 has no influence on palmitoyl CoA synthetase activity.** As palmitoyl CoA synthetase functions in the *de novo* pathway of ceramide synthesis, we examined the influence of PSC 833. The agent was without effect.

**6. PSC 833 has no influence on ceramide synthase activity.** Previous studies have shown that some chemotherapeutic agents, such as daunorubicin, camptothecin, and tumor necrosis factor  $\alpha$ , elicit ceramide generation which contribute to apoptosis. It has generally been thought that ceramide synthase is the regulatory step in the *de novo* pathway of ceramide generation; however, our enzyme assay results showed that PSC 833 treatment had no influence on ceramide synthase activity.

**7. Effect of PSC 833 on serine palmitoyltransferase activity is closely related to its molecular structure.** PSC 833 is a derivative of cyclosporin A, and both have similar molecular structure. The  $\beta$ -ketoamide in cyclosporin A is replaced by a  $\beta$ -hydroxyamide in PSC 833, and PSC 833 has an isopropyl group replacing one of the ethyl groups. It is reported that PSC 833 is a more potent MDR reversing agent compared to cyclosporin

A. To determine whether this difference is related to their actions on ceramide generation and serine palmitoyltransferase activation, we compared the effects of the two derivatives. Cyclosporin A had no effect on serine palmitoyltransferase activity, sphinganine production, or ceramide generation. Therefore, the  $\beta$ -hydroxyamide and ethyl group are essential for the ceramide metabolic response.

### **CONCLUSIONS -**

During the past year I have mastered various lipid analysis techniques using cultured human breast cancer cells as models, and developed and standardized enzyme assays. Our studies demonstrate that PSC 833 induces ceramide generation in breast cancer cell lines via a *de novo* pathway. We have shown, for the first time, that serine palmitoyltransferase is activated by PSC 833. This is important, as etoposide has also been shown to elicit cytotoxicity by activation of serine palmitoyltransferase (23). Furthermore, we have demonstrated a close structure-activity relationship (of PSC 833) for induction of ceramide generation and serine palmitoyltransferase activation. We conclude that PSC 833 increases ceramide generation via a *de novo* pathway, by activation of serine palmitoyltransferase, and this is a key step in the modulation of multidrug resistance and in chemosensitization.

## REFERENCES

1. Endicott JA and Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann Rev Biochem*, 1989; 58, 137-171.
2. Gottesman MM and Pastan I. The multidrug transporter: a double-edged sword. *J Biol Chem*, 1988; 263, 12163-12166.
3. Gottesman MM and Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann Rev Biochem*, 1993; 62, 385-427.
4. Bradley G and Ling V. P-glycoprotein, multidrug resistance and tumor progression. *Cancer Metastasis Rev*, 1994; 13, 223-233.
5. Lavie Y, Cao H-T, Bursten SL, Giuliano AE and Cabot MC. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J Biol Chem*, 1996; 271, 19530-19536.
6. Lucci A, Cho WT, Han T-Y, Giuliano AE, Morton DL and Cabot MC. Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer Res*, 1998; 18, 475-480.
7. Liu Y-Y, Han T-Y, Giuliano AE and Cabot MC. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers Adriamycin resistance in human breast cancer cells. *J Biol Chem*, 1999; 274, 1140-1146.
8. Molina R, Oesterrich S, Zhou JL, Tandon AK, Clark GM, Allred DC, Townsend AJ, Moscow JA, Cowan KH, McGuire WL. Glutathione transferase GST pi in breast tumors evaluated by three techniques. *Dis. Markers*, 1993; 11, 71-82.
9. Morrow C, Chiu J and Cowan K. Posttranscription control of glutathione-S-transferase pi gene expression in human breast cancer cells. *J Biol Chem*, 1992; 267, 10544-10550.
10. Tuccari G, Rizzo A, Giuffre G and Barresi G. Immunocytochemical detection of DNA topoisomerase type 11 in primary breast carcinomas: correlation with clinico-pathological features. *Virchows Arch A Pathol Anat Histopathol*, 1993; 423, 51-55.
11. Slater LM, Sweet P, Stupecky M and Gupta S. Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia in vitro. *J Clin Invest*, 1986; 77, 1405-1408.
12. Twentyman PR. Cyclosporins as drug resistance modifiers. *Biochem Pharmacol*, 1992; 43, 109-117.

13. Lavie Y, Cao H-T, Volner A, Lucci A, Han T-Y, Geffen V, Giuliano AE and Cabot MC. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *J Biol Chem*, 1997; 272, 1682-1687.
14. Cabot MC, Han T-Y and Giuliano AE. The multidrug resistance modulator SDZ PSC 833 is a potent activator of cellular ceramide formation. *FEBS Lett.*, 1998; 431, 185-188.
15. Hwang M, Ahn C, Pine P S, Yin J, Hrycyna C, Licht T and Aszalos A. Effect of combination of suboptimal concentrations of P-glycoprotein blockers on the proliferation of MDR1 gene expressing cells. *Int J Cancer*, 1996; 65, 389-397.
16. Archinal-Mattheis A, Rzepka RW, Watanabe T, Kokubu N, Itoh Y, Combates NJ, Bair KW and Cohen D. Analysis of the interactions of SDZ PSC 833 ([3'-keto-Bmtl]-Val2]-cyclosporine), a multidrug resistance modulator, with P-glycoprotein. *Oncol Res*, 1995; 7, 603-610.
17. Lucci A, Han T-Y, Liu Y-Y, Giuliano AE, Cabot MC. Multidrug resistance modulators and doxorubicin synergize to elevate ceramide levels and elicit apoptosis. *Cancer*, 1999; 86: 300-311.
18. Cabot MC, Giuliano AE, Han T-Y and Liu Y-Y. SDZ PSC 833, the cyclosporine A analog and multidrug resistance modulator, activates ceramide synthesis and induces apoptosis in drug resistant cancer cells. *Cancer Res*, 1999; 59, 880-885.
19. Obeid LM, Linaredic CM, Karolak LA and Hannun YA. Programmed cell death induced by ceramide. *Science*, 1993; 259, 1769-1771.
20. Mathias S, Pena LA and Kolesnick RN. Signal transduction of stress via ceramide. *Biochem J*, 1998; 335, 465-480.
21. Galve-Roperh I, Sanchez C, Cortes ML, del Pulgar TG, Izquierdo M, Guzman M. Anti-tumoral action of ceramide of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med*, 2000, 6: 313-319.
22. Bose R, Verheij M, Halmovitx-Friedman A, Scotto K, Fuks Z and Kolesnick R. Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. *Cell*, 1995; 82, 405-414.
23. Perry DK, Carton J, Shah AK, Meredith F, Uhlinger DJ, Hannun YA. Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *J Biol Chem* 2000; 275: 9078-9084.

## 7. APPENDIX

### KEY RESEARCH ACCOMPLISHMENTS

- mastered techniques of cellular lipid radiolabeling and thin-layer chromatography (TLC), essential for the metabolic studies;
- developed TLC methods for 3-keto-shinganine, sphinganine, and sphingosine analysis;
- developed a method for quantitative analysis of the ceramide generated from different biochemical pathways;
- developed and standardized enzyme assays for palmitoyl coenzyme A synthetase, serine palmitoyltransferase, and ceramide synthase, essential for the enzymology;
- determined that PSC 833 induces apoptosis and reverses multidrug resistance in human breast cancer cells;
- demonstrated that PSC 833 increases ceramide generation in a time- and dose-dependent manner;
- determined that PSC 833 increases ceramide generation via the *de novo* pathway, and not by sphingomyelin hydrolysis;
- demonstrated that PSC 833 activates serine palmitoyltransferase in *in vitro* assays using microsomes obtained from PSC 833-pretreated cells;
- determined that PSC 833 has no influence on palmitoyl CoA synthetase activity;
- determined that PSC 833 has no influence on ceramide synthase activity;
- determined that the effect of PSC 833 on serine palmitoyltransferase activity is closely related with molecular structure, showing a stringent structure-activity relationship.

### REPORTABLE OUTCOME

#### Manuscript in Preparation (*Cancer Research*)

Hongtao Wang, Armando E. Giuliano, and Myles C. Cabot. Activation of serine palmitoyltransferase by PSC 833: insight into mechanisms of ceramide generation and multidrug resistance modulation in breast cancer cells.